DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

QUANTITATIVE PBMC MICROCOCULTURE ASSAY

I. PRINCIPLE

The quantitative PBMC micrococulture assay estimates the number of infectious units of HIV per million mononuclear cells (IUPM) in peripheral blood mononuclear cells (PBMC). The greater the number of cells needed to produce a positive result, the lower the virus load in the PBMC. The assay, as described in detail below, is performed in duplicate in a 24-well tissue culture plate using six 5-fold dilutions, beginning with one million patient PBMC. Each sample of patient cells is cocultured with PHA-stimulated normal donor PBMC for 14 days. The supernatant from each individual well is assayed for viral expression of HIV-1 p24 antigen by the standard HIV p24 EIA assay.

II. SPECIMEN REQUIREMENTS

The assay utilizes PBMC isolated from heparinized, ACD, CPD or EDTA anticoagulated peripheral blood (usually 10 mL is required for adults or children and 2 mL from infants). The blood must be kept at room temperature until processing and should be processed within 30 hours of collection.

III. REAGENTS

All reagents are prepared using deionized water, reagent grade I.

Sterile Phosphate Buffered Saline (PBS) or sterile Hank's Balanced Salt Solution (HBSS) without calcium or magnesium. Store 10X buffer at room temperature and 1X buffer at 4^0C. Note manufacturer's outdate or discard one week after opening.

Sterile Ficoll-Hypaque or Lymphocyte Separation Medium (LSM) - Store at room temperature in the dark. Note manufacturer's outdate and date opened.

Penicillin - available in $5x10^6$ unit vials. Store at room temperature. Observe manufacturer's outdate.

a. Add 25 mL of sterile water to the vial. Mix until contents are dissolved. Final concentration = 200,000 units/mL

b. Divide into 0.32 mL aliquots in sterile 1.5 mL microcentrifuge tubes and freeze at -20^0C in a labeled box. Label with a 1 year outdate.

Gentamicin - available in 50 mg/mL bottles. Open bottles under laminar flow hood only; divide into 0.640 mL aliquots in sterile 1.5 mL microcentrifuge tubes. Store unopened bottles at room temperature; store aliquots at 4^0C. Outdates one month after opening.

Fetal Bovine Serum (FBS) - available in 500 mL sterile bottles from various manufacturers. Store frozen at -20^0C. Note manufacturer's outdate. When needed, rapidly thaw a bottle in a 37^0C water bath, then heat-inactivate in a 56^0C water bath for 30 minutes with occasional shaking. The level of H_2O in the water bath should be as high as the level of the serum in the bottle. Store at 4^0C after thawing. Heat-inactivated FBS has a one month outdate.

RPMI 1640 medium with L-glutamine (2 mM) - Store at 4^0C and observe manufacturer's outdate.

IL-2 (interleukin-2) - available in 50 mL bottles from Boehringer Mannheim at a discounted price for labs within the ACTG. Store at -20^0C. Note manufacturer's outdate. As needed, thaw a 50 mL bottle (freeze the remaining 25 mL).

Basic Medium:

To make 620 mL:

a. Add 120 mL FBS to 500 mL of RPMI 1640 medium with L-glutamine. Final concentration (120/620) is approximately 20%.

b. Add 310 µL stock penicillin. (Concentration of penicillin used is $5x10^6$ units/25 mL or 200,000 units/mL; 0.31 mL of 200,000 units/mL = 62,000 units and 62,000 units/620 mL final volume of medium=100 units/mL for final concentration).

c. Add 620 µL Gentamicin. (Concentration of Gentamicin used is 50 mg/mL or 50 µg/µL; 620 µL of 50 µg/µL = 31,000 µg and 31,000 µg/620 mL final volume of medium = 50 µg/mL for final concentration).

Store Basic Medium at 4^0C for up to 1 month.

Growth Medium - also called IL-2 Medium or T-Cell Growth Factor (TCGF) Medium.

To make 500 mL:

a. 475 mL Basic Medium.

b. 25 mL IL-2. (Final concentration = 25 mL/500 mL = 5%.)

Store Growth Medium at 4^0C for up to 1 month. Growth Medium should warmed before use.

Trypan Blue Stain - this stains non-viable cells dark blue, and is used to determine the viable cell count of a culture. Prepare a 0.4% solution by adding 0.4 gm Trypan Blue (available from Sigma) and 1 mL Glacial Acetic Acid to 99 mLs distilled H_20 or saline. After dissolving, filter solution through Whatman filter paper or a 0.45 µ filter.

PHA-stimulated uninfected donor PBMCs - see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC).

IV. EQUIPMENT AND SUPPLIES

Gloves
Disposable lab coat
Accuspin tubes with Ficoll, available from Sigma in 12 mL or 50 mL size tubes.
Sterile 15 and 50 mL conical tubes.
Sterile 2, 5, 10, and 25 mL pipettes.
Hemacytometer.
Sterile 24-well tissue culture plates.
Sterile 500 mL bottles.
Sterile 1.5 and 0.5 mL microcentrifuge tubes.
20 µL, 200 µL, and 1000 µL pipetteman.
Sterile 200 µL and 1000 µL pipette tips.
Bleach (household bleach diluted 1/100 with tap water).
Laminar flow hood (Class 2 biosafety hood).
Centrifuge capable of speeds up to 1500 x g and equipped with a horizontal rotor and O- ring sealed safety cups.
Compound microscope.
CO_2 incubator (37 ± 1^0C with humidity).
37^0C and 56^0C water baths.
Pipette aid.

V. PROCEDURE

1. Log patient information into the lab computer and label specimen with the assigned specimen number.

2. Obtain PBMC from whole blood. See Qualitative PBMC Macrococulture Assay.

NOTE: SUBSEQUENT PROCEDURES SHOULD BE PERFORMED IN A CLASS 2 BIOSAFETY LAMINAR FLOW HOOD USING STERILE TECHNIQUE AND ADHERING TO CDC/NIH STANDARDS (INCLUDING USE OF GLOVES AND LAB COATS).

3. Make six 5-fold serial dilutions as follows:

   a. For the first tube in the series, start with $3x10^6$ patient cells in 3 mL of Growth Medium. A minimum of $2.7x10^6$ patient PBMC (in 2.7 mL) are required for the following scheme. See note below if minimum requirement is not met.

   b. Transfer 0.6 mL of the cell suspension from step 1. to the next tube in the series containing 2.4 mL of Growth Medium. Mix.

   c. Continue as in step 2., using a new pipette tip for the removal from each tube, to make 6 dilutions. The resulting dilution scheme is 1:1, 1:5, 1:25, 1:125, 1:625, 1:3125. The resulting counts per mL will be 1,000,000, 200,000, 40,000, 8,000, 1600, and 320 patient PBMC per mL.

   NOTE: If fewer than $2.7x10^6$ but more than $2x10^6$ patient PBMC are recovered from a sample, the first tube should be adjusted to contain $2.0x10^6$ PBMC in 2.0 mL of Growth Medium. Proceed with step 2. above.

   If fewer than $2.0x10^6$ PBMC are recovered from a sample, dilute the total number of cells in 3.0 mL of Growth Medium and proceed to make the 5-fold dilutions from this starting concentration. In the computer, it will be necessary to enter the new estimated concentration per mL (total number of PBMC recovered divided by 3) rather than defaulting to $1x10^6$ for the number of cells in the 1:1 dilution.

4. Pipette 1.5 mL of sterile water into each of 4 corner wells of an appropriately labeled 24-well plate. In duplicate, pipette 1 mL of each of the 6 patient cell dilutions into respective wells. Store remaining patient PBMC according to each protocol (viable PBMC, pellets, etc.). (See “Specimen Processing” and “Specimen Storage Recommendations”.)

   NOTE: In the case of fewer than $2.7x10^6$ but $>2.0x10^6$ PBMC, the first tube will only have 1.4 mL and can only be tested in singleton. The first set of wells will include only one well with a single sample of $1x10^6$ PBMC.

5. Prepare donor cells at a concentration of $1x10^6$ cells/mL**.** One mL will be needed for each well of the plate (12) plus extra to facilitate pipetting.

   a. Centrifuge $13x10^6$ PHA-stimulated donor cells at 400 x g for 10 minutes.

   b. Decant supernatant.

   c. Mix pellet and suspend in 13 mL of Growth Medium.

6. Add 1 mL of donor cell preparation to each well.

7. Incubate at 37^0C, 5% CO_2 with humidity.

8. Feed and sample as follows:

   a. Remove 1 mL of supernatant from each well without disturbing the cells.

      1) Day 7 - discard supernatant. (These may be saved if desired, in case of trouble with the assay, but may only be used to troubleshoot the assay and not to calculate an IUPM.)

      2) Day 14 - save supernatant from each well, frozen in appropriately labeled tubes for HIV p24 antigen testing.

   b. On day 7 add 1 mL of 0.5 X 10^6 PHA-stimulated donor cells to each well.

   Prepare donor cells for feeding at a concentration of $0.5x10^6$ cells/mL**.** One mL will be needed for each well plus extra to facilitate pipetting. NOTE: This concentration differs from that used to set up the initial assay.

      1) Centrifuge $7x10^6$ donor cells at 400 x g for 10 minutes.
      2) Decant supernatant.
      3) Mix pellet and suspend in 14 mL of Growth Medium.

9. Culture wells to be sampled are listed in a "sampling list" which is computer generated each day from the laboratory management program. Supernatant aliquots are saved in sterile tubes and stored at -20^0C or -70^0C until assayed for HIV p24 antigen level.

10. IUPM is calculated by the method of maximum likelihood from the pattern of positive culture wells in the assay. A well is scored positive if the VQA corrected value 30 pg/mL.

11. At the end of culture, save the appropriate samples according to each protocol (supernatant, cells, PLP, etc.). (See "Specimen Processing" and "Specimen Codes".)

VI. REPORTING

The IUPM calculated by the computer is reported. A low Goodness of Fit (< 0.05) may invalidate the result.

VII. REFERENCES

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